Mature dendritic cells pulsed with exosomes stimulate efficient cytotoxic T-lymphocyte responses and antitumour immunity

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Summary

Exosomes (EXO) derived from dendritic cells (DC), which express major histocompatibility complex (MHC) and costimulatory molecules, have been used for antitumour vaccines. However, they are still less effective by showing only prophylatic immunity in animal models or very limited immune responses in clinical trials. In this study, we showed that ovalbumin (OVA) protein-pulsed DC (DC_{OVA})-derived EXO (EXO_{OVA}) displayed MHC class I–OVA I peptide (pMHC I) complexes, CD11c, CD40, CD80, CCR7, DEC205, Toll-like receptor 4 (TLR4), TLR9, MyD88 and DC-SIGN molecules, but at a lower level than DC_{OVA}. EXO_{OVA} can be taken up by DC through LFA-1/CD54 and C-type lectin/mannose (glucosamine)-rich C-type lectin receptor (CLR) interactions. Mature DC pulsed with EXO_{OVA}, which were referred to as mDC_{EXO}, expressed a higher level of pMHC I, MHC II, and costimulatory CD40, CD54 and CD80 than DC_{OVA}. The mDC_{EXO} could more strongly stimulate OVA-specific $CD8^+$ T-cell proliferation *in vitro* and *in vivo*, and more efficiently induce OVA-specific cytotoxic T-lymphocyte responses, antitumour immunity and $CD8^+$ T-cell memory *in vivo* than EXO_{OVA} and DC_{OVA}. In addition, mDC_{EXO} could also more efficiently eradicate established tumours. Therefore, mature DC pulsed with EXO may represent a new, highly effective DC-based vaccine for the induction of antitumour immunity.

Keywords: antitumour immunity; cytotoxic T lymphocyte; dendritic cell; exosome; vaccine

Introduction

Dendritic cells (DC) process exogenous antigens in endosomal compartments, such as multivesicular endosomes,[1] that can fuse with plasma membrane, thereby releasing antigen-presenting vesicles called 'exosomes'.[2–4] These exosomes (EXO) are 50- to 90-nm diameter vesicles containing antigen-presenting molecules [major histocompatibility complex (MHC) class I, MHC class II, CD1, heatshock protein (hsp 70–90)], tetraspan molecules (CD9, CD63, CD81), adhesion molecules (CD11b, CD54) and CD86 costimulatory molecules,[5–7] i.e. the necessary machinery required for generating potent immune responses. Zitvogel *et al.* first reported that DC-derived EXO can be successfully used as vaccines for the eradication of tumours in animal models.[4] Clinical grade EXO derived from DC can be obtained.[8] However, immunotherapy using DC-derived EXO has its limitations, the greatest being the limited availability of identified tumour antigens. Alternatively, tumour-cell-derived EXO were found to carry tumour antigens that were capable of triggering effective antitumour immune responses.[9–11] Therefore, tumour-derived EXO, such as those derived from tumour ascites,[12] offer a new source of cell-free EXO for

Abbreviations: CFSE, 5-carboxy-fluorescein diacetate succinimidyl ester; CTL, cytotoxic T lymphocyte; DC, dendritic cells; DC_{OVA}, OVA-pulsed DC; EXO, exosome; FITC, fluorescsein isothiocyanate; GM-CSF, granulocyte–macrophage colony-stimulating factor; hsp, heatshock protein; IFN-γ, interferon-γ; IL-4, interleukin-4; imDC, immature DC; KO, knockout; mDC, mature DC; MHC, major histocompatibility complex; OVA, ovalbumin; PBS, phosphate-buffered saline; pMHC I, peptide–MHC class I; TCR, T-cell receptor; TLR, Toll-like receptor.

cancer vaccines. More recently, EXO-based vaccines have been confirmed to induce antitumour immunity in different animal models.[13–16] However, in most of these studies, EXO-based vaccines can only induce prophylactic antitumour immune responses, they cannot cure established tumours. In a recent phase I clinical trial using EXO vaccine, there was no tumour-specific T-cell response detected in the peripheral blood of all 15 melanoma patients and only one patient exhibited a partial response to tumour growth.[17] In another phase I clinical trail using EXO vaccine, a delayed-type hypersensitivity reaction against MAGE peptides was only detected in three of nine patients with advanced non-small cell lung cancers.[18] Therefore, the efficiency of the EXO-based vaccine approach needs to be further improved.

Exosomal peptide–MHC I and II complexes (pMHC I or II) are functional but need to be transferred to DC to promote T-cell activation leading to tumour eradication.[14,19,20] Therefore, the potential pathway of *in vivo* EXO-mediated antitumour immunity may be through uptake of EXO by immature DC (imDC), which in turn stimulate antigen-specific T lymphocytes via the pMHC complexes and costimulatory molecules on DC that have taken up EXO. We have previously shown that the efficiency of antitumour immunity derived from DC-based vaccines depends upon the maturation stage of DC, i.e. high efficiency from mature DC and low efficiency from less mature DC.[21] Therefore, the lower efficiency of EXO vaccines may be the result of the uptake of EXO *in vivo* by imDC. To improve the efficiency of antitumour immunity, CpG adjuvants, which bind to Toll-like receptor 9 (TLR9) on DC and which cause DC maturation and activation,[22] have been applied *in vivo* in conjuction with EXO to enhance EXO-induced cytotoxic T-lymphocyte (CTL) responses.[15] Alternatively, we hypothesized that DC with EXO uptake *in vitro* may also become a new effective approach for the induction of antitumour immunity.

In this study, we first systematically and phenotypically characterized EXO derived from DC. Since the DC that take up EXO gain the antigen-specificity from those EXO, they become immunogenic and are capable of stimulating antigen-specific CTL responses. To prove the above hypothesis that DC with EXO uptake may induce effective antitumour immunity, we investigated (i) the efficiency of EXO uptake by immature and mature DC, (ii) the molecular mechanism for EXO uptake by DC, and (iii) the antitumour immunity derived from EXO-pulsed DC vaccines.

Materials and methods

Reagents, cell lines and animals

Ovalbumin (OVA) protein was obtained from Sigma (St Louis, MO). OVA I (SIINFEKL) peptide[23,24] and *Mut*I (FEQNTAQP) peptide, specific for an irrelevant 3LL lung carcinoma,[25] were synthesized by Multiple Peptide Systems (San Diego, CA). Biotin-labelled and fluorescein isothiocyanate (FITC)-labelled antibodies specific for H-2K^b (AF6-88.5), Ia^b (AF6-120.1), CD4 (GK1.5), CD8 (53-6.7), CD11c (HL3), CD40 (IC10), CD54 (3E2), CD80 (16-10A1), CD44 (IM7), MyD88 and CCR7 (4B12) were obtained from Pharmingen Inc (Mississauga, Ont., Canada). The anti-H-2K^b/OVA I (pMHC I) complex antibody was obtained from Dr Germain (National Institutes of Health, Bethesda, MD).[26] Phycoerythrin-labelled H-2K^b/OVA I tetramer antibody was obtained from Beckman Coulter (Mississauga, Ont., Canada). Biotin-labelled TLR4 and TLR9 antibodies were obtained from eBioscience (San Diego, CA). The anti-LFA-1, anti-K^b, anti-Ia^b and anti-DEC205 antibodies, and the CTL-associated antigen (CTLA-4/Ig) fusion protein, the recombinant mouse interleukin-4 (IL-4) and granulocyte–macrophage colony-stimulating factor (GM-CSF) were purchased from R & D Systems Inc. (Minneapolis, MN). The cytochalasin D, D-mannose, D-glucose, D-fucose and D-glucosamine were purchased from Sigma. The 5-carboxy-fluorescein diacetate succinimidyl ester (CFSE) was obtained from Molecular Probes (Eugene, OR). The highly lung metastatic BL/6-10 and the OVA-transfected BL6-10 (BL6-10$_{OVA}$) melanoma cell lines were generated in our own laboratory.[27] The mouse EL4 and OVA-transfected EL4 (EG7) thymoma cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Female C57BL/6 mice (B6; CD45.2$^+$), C57BL/6.1 mice (B6.1; CD45·1$^+$), and OVA-specific T-cell receptor (TCR) transgenic OT I and OT II mice, and H-2K^b, CD4, CD8, CD54 and CD80 gene knockout (KO) mice on a C57BL/6 background were all obtained from the Jackson Laboratory (Bar Harbor, MA). All mice were maintained in the animal facility at the Saskatoon Cancer Center and treated according to the animal care committee guidelines of the University of Saskatchewan.

Generation of bone marrow-derived DC

The generation of bone marrow-derived imDC under low-dose GM-CSF (2 ng/ml) and of mature DC (mDC) under high-dose GM-CSF/IL-4 (20 ng/ml) has been described previously.[21] DC at day 6 of culture were further pulsed with OVA protein (0·3 mg/ml) in fetal calf serum-free AIM-V medium (Gibco, Burlington, Canada) for overnight culture and termed DC$_{OVA}$. DC derived from H-2K^b KO mice were termed DC(K$^{b-/-}$).

Generation and purification of EXO

EXO were isolated as described previously.[3,4] Briefly, culture supernatants of mDC$_{OVA}$ cultured overnight in fetal calf serum-free AIM-V medium containing OVA (0·3 mg/ml) were subjected to four successive centrifugations

at 300 ***g*** for 5 min to remove cells, 1200 ***g*** for 20 min and 10 000 ***g*** for 30 min to remove cellular debris and 100 000 ***g*** for 1 hr to pellet the EXO. The EXO pellets were washed twice in a large volume of phosphate-buffered saline (PBS) and recovered by centrifugation at 100 000 ***g*** for 1 hr. The amount of exosomal protein recovered was measured using a Bradford assay (Bio-Rad, Richmond, CA). EXO derived from mDC_{OVA} of wild-type C57BL/6 and C57BL/6.1 mice were termed EXO_{OVA} and $EXO_{6.1}$, respectively. EXO derived from mDC_{OVA} of H-$2K^b$, CD54, CD80 KO mice were termed ($K^{b-/-}$)EXO, ($CD54^{-/-}$)EXO and ($CD80^{-/-}$)EXO, respectively. To obtain CFSE-labelled EXO_{CFSE}, mDC were stained with 0·5 μM CFSE at 37° for 20 min and washed three times with PBS,[28,29] then pulsed with OVA protein in AIM-V serum-free medium for overnight culture. The CFSE-labelled EXO_{CFSE} were then harvested and purified from the culture supernatants as described above.

Phenotypic characterization of DC and EXO

For phenotypic analysis of DC, both $imDC_{OVA}$ and mDC_{OVA} were stained with a panel of biotin-labelled and FITC-labelled antibodies and were analysed by flow cytometry. For phenotypic analysis of EXO, EXO_{OVA} (25–40 μg) were incubated with a panel of FITC-conjugated antibodies on ice for 30 min, and then analysed by flow cytometry as previously described.[7] To determine the optimal voltage suitable for EXO analysis, 4·5-μm diameter Dynal M450 beads (DYNAL Inc, Lake Success, NY) were used as size controls for the flow cytometric analysis[7] using FACScan (Coulter EPICS XL, Beckman Coulter, San Diego, CA). For analysis of the expression of intracellular molecules such as TLR9 and MyD88, DC and EXO were rendered permeable using Cytofix/Cytoperm Plus Kit (Pharmingen Inc) according to the company's protocol before antibody staining. Isotype-matched biotin-labelled or FITC-conjugated antibodies were used as controls.

Preparation of T cells

Naive OVA-specific T cells were isolated from OVA-specific TCR transgenic OT I and OT II mouse spleens, and enriched by passage through nylon wool columns (C & A Scientific, Manassas, VA). OT II $CD4^+$ and OT I $CD8^+$ T cells were then purified by using anti-mouse CD8 (Ly2) and CD4 (L3T4) paramagnetic beads (DYNAL Inc.)[27] to remove $CD8^+$ and $CD4^+$ T cells and yield populations that were ∼95% $CD4^+$ $V\alpha2V\beta5^+$ and $CD8^+$ $V\alpha2V\beta5^+$ T cells, respectively.

EXO uptake by DC

To test EXO transferred onto DCs, mDC were cocultured with EXO_{CFSE} (10 μg/1×10^6 DC), and then CFSE-positive cells were detected at different time-points for up to 10 hr by fluorescence microscopy. To investigate the rate of decay, DCs incubated with EXO_{CFSE} for 4 hr were washed twice with PBS, cultured further in culture medium, and then examined at different time-points for up to 72 hr by fluorescence microscopy. To further confirm EXO uptake, mDC and imDC were cocultured with EXO_{CFSE} or $EXO_{6.1}$ and then analysed for CFSE staining and expression of CD45.1 molecule, respectively, by flow cytometry. To investigate the molecular mechanisms involved in EXO uptake, mDC($K^{b-/-}$) were incubated with a panel of antibodies specific for H-$2K^b$, Ia^b, LFA-1 and DEC205 (15 μg/ml), the fusion protein CTLA-4/IgG (10 μg/ml), an inhibitor of actin polymerization cytochalasin D (15 μg/ml), D-mannose, D-glucose, D-fucose and D-glucosamine (5 mM), and ethylene diamine tetraacetic acid (EDTA; 50 mM), respectively, on ice for 30 min before and during coculturing with EXO_{OVA}. Both mDC and imDC were cocultured with EXO_{OVA} (10 μg/1×10^6 DC) in 0·5–1 ml AIM-V medium at 37° for 4 hr, washed twice with PBS and termed mDC_{EXO} and $imDC_{EXO}$.

In vitro *T-cell proliferation assay*

To assess the functional effect of DC-derived EXO, we performed an *in vitro* $CD8^+$ T-cell proliferation assay. EXO_{OVA} (10 μg/ml) and their two-fold dilutions were cultured with a constant number of naive OT I $CD8^+$ T cells (1×10^5 cells/well). To test whether pMHC I complexes of EXO_{OVA} taken up by DC were functional, mDC ($0{\cdot}3 \times 10^5$ cells/well) and imDC ($0{\cdot}3 \times 10^5$ cells/well) were cocultured with EXO_{OVA} and their two-fold dilutions for 4 hr, and then a constant number of naive OT I $CD8^+$ T cells (1×10^5 cells/well) was added into each well. To examine the molecular mechanism, before OT I $CD8^+$ T cells were added, a panel of reagents including anti-H-$2K^b$ and LFA-1 antibodies, and CTLA-4/Ig fusion protein (each 10 μg/ml), a mixture of the above reagents (as mixed reagents) and a mixture of isotype-matched irrelevant antibodies (as control reagents) were added to the culture of mDC and EXO_{OVA}, respectively. After culturing for 48 hr, thymidine incorporation was determined by liquid scintillation counting.[25]

Tetramer staining and ELISPOT assays

C57BL/6 or CD4 KO mice were intravenously (i.v.) immunized with EXO_{OVA} (10 μg/mouse) and irradiated (4000 rad) DC_{OVA}, mDC_{EXO} and $imDC_{EXO}$ ($0{\cdot}5 \times 10^6$ cells/mouse), respectively. In one set of experiments, the blood samples were incubated with 10 μl of phycoerythrin-conjugated H-$2K^b$/$OVA_{257–264}$ tetramer (Beckman Coulter, Mississauga, Ont., Canada) and FITC-conjugated anti-CD8 (53-6.7) for 30 min at room tem-

perature. The erythrocytes were then lysed using lysis/fixed buffer (Beckman Coulter) and analysed by flow cytometry. In another set of experiments, the above immunized mice were i.v. boosted with irradiated DC_{OVA} ($0{\cdot}5 \times 10^6$) 3 months after immunization, the blood samples were analysed by flow cytometry 4 days after the boost. In ELISPOT assay[30] splenocytes (1×10^6 cells) harvested from mice 6 days after the primary immunization were seeded into each well of filtration plates (96-well plates; Millipore, Bedford, MA) in the absence (as control) or presence of OVA I (2 μM), which were previously coated with purified anti-interferon-γ (IFN-γ) antibody for 24 hr and blocked with 10% FCS. The plates were then incubated at 37° for 24 hr. After washing, biotin-conjugated anti-IFN-γ monoclonal antibodies were added and incubated for 2 hr at room temperature. The plates were then washed three times with distilled water. Streptavidin–alkaline phosphatase (Invitrogen, Carlsbad, CA) was added, and the plates were incubated for 1–2 hr at room temperature. After three washes with distilled water, the alkaline phosphatase substrate BCIP/NBT (Sigma) was added, and the colour was developed according to the manufacturer's instructions. Spots were counted under a microscope.

Animal studies

To examine protective antitumour immunity, wild-type C57BL/6, CD4 KO or CD8 KO mice ($n = 8$) were injected i.v. with EXO_{OVA} (10 μg/mouse), and irradiated (4000 rad) DC_{OVA} ($0{\cdot}05 \times 10^6$–$0{\cdot}5 \times 10^6$ cells/mouse), mDC_{EXO} ($0{\cdot}05 \times 10^6$–$0{\cdot}5 \times 10^6$ cells/mouse) and $imDC_{EXO}$ ($0{\cdot}5 \times 10^6$ cells/mouse), respectively. The immunized mice were i.v. challenged with $0{\cdot}5 \times 10^6$ BL6-10_{OVA} 6 days or 3 months after immunization. To examine the therapeutic effect on established tumours, wild-type C57BL/6 mice ($n = 15$) were first injected i.v. with $0{\cdot}5 \times 10^6$ BL6-10_{OVA} tumour cells. After 5 days, the mice were i.v. immunized with irradiated DC_{OVA} and mDC_{EXO} ($1{\cdot}0 \times 10^6$ cells/mouse). The mice were killed 4 weeks after tumour cell injection and the metastatic lung tumour colonies were counted in a blind fashion. Metastases on freshly isolated lungs appeared as discrete black pigmented foci that were easily distinguishable from normal lung tissues and were confirmed by histological examination. Metastatic foci too numerous to count were assigned an arbitrary value of > 100.[27] To further confirm the therapeutic effect on established tumours, C57BL/6 mice ($n = 10$) were also injected subcutaneously (s.c.) with $0{\cdot}3 \times 10^6$ BL6-10_{OVA} tumour cells. After 4 days, when tumours became palpable (~3 mm in diameter), mice were s.c. immunized with irradiated DC_{OVA} and mDC_{EXO} ($1{\cdot}0 \times 10^6$ cells/mouse). Animal tumour growth was monitored daily using a calliper for up to 4 weeks; for humanitarian reasons, all mice with 1·5-cm diameter tumours were killed.

Results

Phenotypical characterization of DC and EXO

The imDC displayed low expression of MHC class II (Ia^b), costimulatory molecule CD80 and chemokine receptor CCR7 and were deficient in CD40 expression (Fig. 1), each of which plays a critical role in T-cell activation. The mDC exhibited higher expression of the above molecules compared with the imDC (Fig. 1). Both imDC and mDC displayed expression of CD11c, adhesion molecule CD54, TLR4, TLR9, MyD88, C-type lectins DEC205 with ligand specificity for mannose and DC-SIGN with ligand specificity for mannan, Le^X, etc. They expressed similar amounts of the H-$2K^b$/OVA I peptide (pMHC I) complexes after pulsing with OVA protein. The expression of pMHC I, MHC class II (Ia^b), CD11c, CD40, CD54, CD80, CCR7, TLR4, TLR9, MyD88, DEC205 and DC-SIGN were also detected on EXO_{OVA}. However, the expression of the above molecules, except for TLR4, was at a relatively lower level than mDC_{OVA} (Fig. 1).

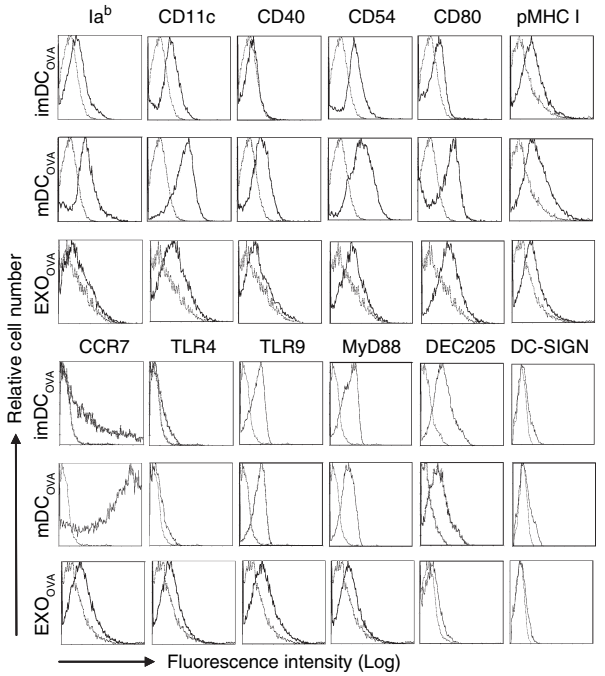

Figure 1. Phenotypic analysis of DC and DC-derived exosomes. BM-derived mDCs, imDCs and mDC-derived exosomes (solid lines) were stained with a panel of antibodies, and then analysed by flow cytometry. These cells and exosomes were also stained with isotype-matched irrelevant antibodies, respectively, and employed as control populations (thin dotted lines). One representative experiment of two is displayed.

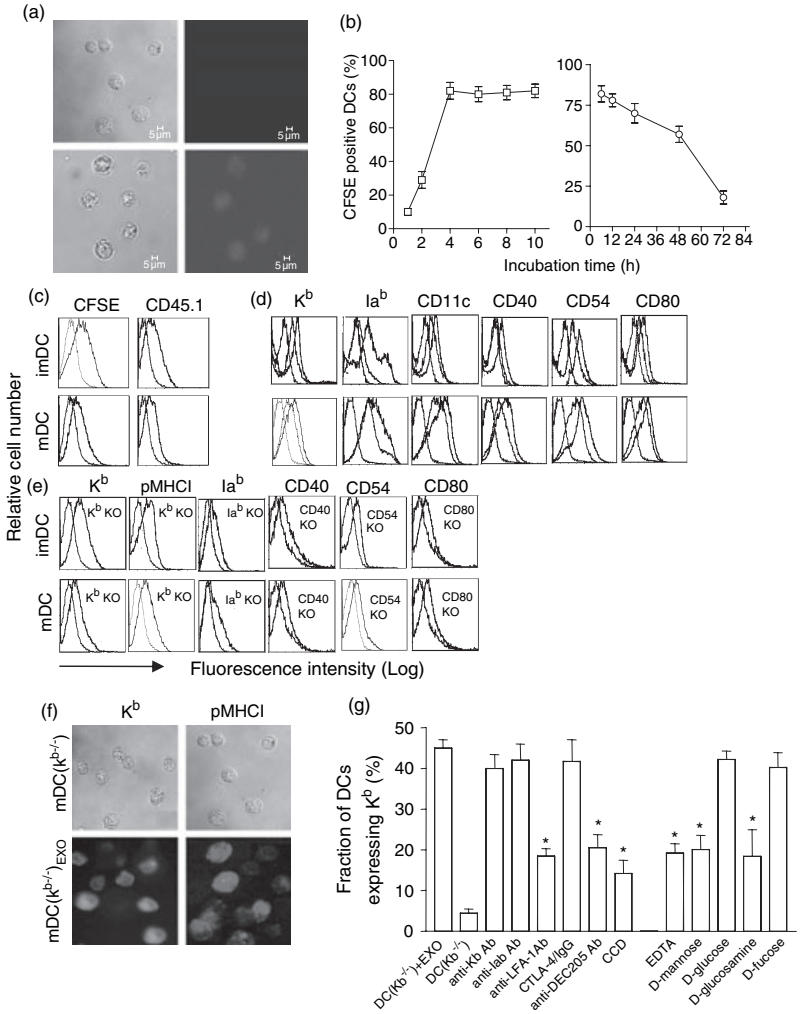

Figure 2. Exosome uptake by DC. (a) To test EXO transferred onto DC, mDCs were cocultured with EXO_{CFSE} for 4 hr, and then examined by fluorescence microscopy. (b) The CFSE-positive cells were detected at different time-points for up to 10 hr after incubation with EXO_{CFSE} by fluorescence microscopy. To investigate the rate of decay, DCs incubated with EXO_{CFSE} for 4 hr, and further cultured in the culture medium. The CFSE-positive cells were then examined and detected at different time-points for up to 72 hr of culturing by fluorescence microscopy. (c) Both mDCs and imDCs with (thick solid lines) and without (thin dotted lines) uptake of EXO_{CFSE} and $EXO_{6.1}$ were analysed for CFSE and CD45.1 expression by flow cytometry. (d) Both mDCs and imDCs with (thick solid lines) and without (thick dotted lines) uptake of EXO_{OVA} were analysed for the expression of a panel of surface molecules by flow cytometry. Irrelevant isotype-matched antibodies were used as controls (thin dotted lines). (e) Both mDCs and imDCs derived from gene KO mice with (thick solid lines) and without (thin dotted lines) uptake of EXO_{OVA} were analysed for expression of a panel of surface molecules including H-$2K^b$, pMHC I, Ia^b, CD40, CD54 and CD80, respectively, by flow cytometry. (f) mDCs derived from H-$2K^b$ gene KO mice with and without uptake of EXO_{OVA} were analysed by fluorescent microscopy. (g) To investigate the molecular mechanisms involved in EXO taken up by DC, mDC($K^{b-/-}$) were incubated with a panel of anti-H-$2K^b$, Ia^b, LFA-1 and DEC205 antibodies, the fusion protein CTLA-4/IgG, cytochalasin D, D-mannose, D-glucose, D-fucose, D-glucosamine and EDTA, respectively, on ice for 30 min before and during coculturing with EXO_{OVA}. DCs were then analysed for expression of H-$2K^b$ by flow cytometry. $^*P < 0{\cdot}05$ versus cohorts without adding any neutralizing reagent (Student's *t*-test).[45] One representative experiment of two is displayed.

DC uptake exosomal molecules

To confirm EXO transferred to DCs, mDC were cocultured with EXO_{CFSE} for 4 hr, and then examined by fluorescence microscopy. As shown in Fig. 2(a), the CFSE-positive cells were seen under fluorescence microscopy. Actually, the CFSE-positive cells (12%) were also detectable even after 1 hr of incubation (Fig. 2b). The CFSE-positive cells (82%) reached a plateau after 4 hr of incubation. To assess the rate of decay, DCs incubated with EXO_{CFSE} for 4 hr were further cultured in the culture medium, and then examined by fluorescence microscopy at different time-points. As shown in Fig. 2(b), the number of CFSE-positive DCs decreased with time. The

CFSE-positive DCs (18%) were still detectable 72 hr after culturing, indicating that the EXO that had been taken up on DCs was quite stable. To further confirm EXO uptake, mDC and imDC were cocultured with EXO_{CFSE} or $EXO_{6.1}$ and then analysed for CFSE staining and expression of CD45.1, respectively, by flow cytometry. To assess EXO uptake by DC, mDC and imDC were incubated with CFSE-labelled EXO_{CFSE} and then analysed by flow cytometry. As shown in Fig. 2(c), the CFSE dye was detectable on both mDC and imDC, indicating that DC can absorb EXO. To further confirm it, both mDCs and imDCs were also incubated with $EXO_{6.1}$ expressing the CD45.1 molecule. As shown in Fig. 2(c), both mDCs and imDCs acquired CD45.1 after incubation with $EXO_{6.1}$. Furthermore, other EXO molecules, such as MHC class I and II, CD11c, CD40, CD54 and CD80 molecules, could also be transferred onto both imDC and mDC (Fig. 2d). To confirm the acquisition, we incubated EXO with DC derived from C57BL/6 mice with different KO. As shown in Fig. 2(e), the original mDC and imDC derived from gene KO mice did not express H-$2K^b$, pMHC.I, Ia^b, CD40, CD54 and CD80, respectively. However, each of them was displayed on DC after incubation with EXO_{OVA}, indicating that an increased expression of the above molecules was the result of the acquisition of EXO molecules by DC. The transfer of exosomal pMHC I onto DC, which is critical in the stimulation of OVA-specific CTL responses, was also confirmed by fluorescence microscopy (Fig. 2f).

EXO uptaken by DC is mediated by LFA-1/CD54 and C-type lectin/C-type lectin receptor interactions

To elucidate the molecular mechanisms involved in EXO uptake, we performed an inhibition assay using a panel of blocking reagents. As shown in Fig. 2(g), EXO uptake by DC was significantly decreased by blocking with the anti-LFA-1 and anti-DEC205 antibodies ($P < 0{\cdot}05$), but not with the anti-H-$2K^b$ and anti-Ia^b antibodies, and the CTLA-4/Ig fusion protein, indicating that LFA-1/CD54 and C-type lectin/mannose-rich C-type lectin receptor (CLR) interactions are involved in EXO uptake. In addition, EXO taken up by DC was also significantly reduced ($P < 0{\cdot}05$) after treatment with cytochalasin D (an inhibitor of actin polymerization), indicating that the actin cytoskeleton is crucial for EXO uptake. Since the interaction of C-type lectin and CLR is calcium-dependent[31] we then used EDTA, which is capable of chelating calcium ions. As shown in Fig. 2(g), EDTA (50 mM) significantly reduced EXO uptake by DC ($P < 0{\cdot}05$), confirming that EXO uptake by DC is mediated with C-type lectin/CLR interactions. To further confirm the involvement of C-type lectin/mannose-rich CLR interaction in EXO uptake, we used a panel of monosaccharides in the blocking test. Interestingly, both D-mannose and D-glucosamine, but not D-glucose and D-fucose, significantly reduced EXO uptake ($P < 0{\cdot}05$), indicating that EXO taken up by DC is mediated at least partially by interaction between C-type lectin and mannose/glucosamine-rich CLR.

EXO-pulsed DC stimulate naive $CD8^+$ T-cell proliferation *in vitro*

Since EXO harbour immune molecules, they have a potent effect in the stimulation of $CD8^+$ T cells.[32] Our data showed that EXO_{OVA} stimulated OT I $CD8^+$ T-cell proliferation *in vitro*, but with much less efficiency than DC_{OVA}, mDC_{EXO} and $imDC_{EXO}$ (Fig. 3a), indicating that EXO require DC to more efficiently activate naive $CD8^+$

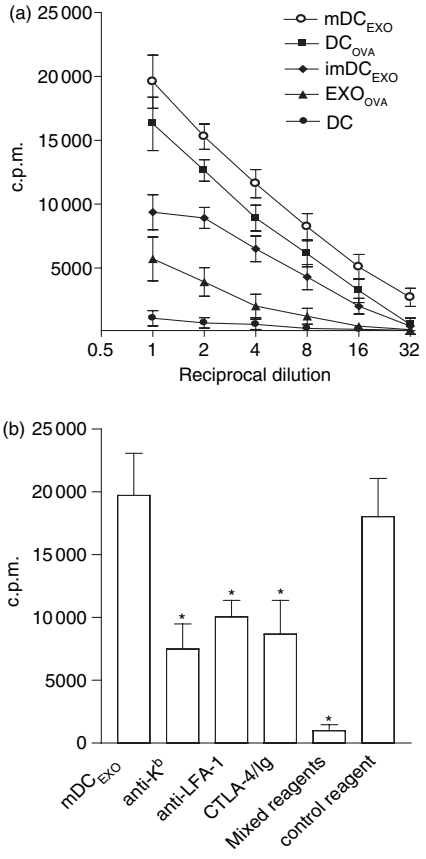

Figure 3. Stimulation of T-cell proliferation *in vitro*. (a) *In vitro* $CD8^+$ cell proliferation assay. EXO_{OVA} (10 μg/ml), DC_{OVA}, mDC_{EXO} and $imDC_{EXO}$ ($0{\cdot}3 \times 10^5$ cells/well) and their two-fold dilutions were cocultured with a constant number of OT I $CD8^+$ T cells (1×10^5 cells/well). After 2 days, the proliferation response of $CD8^+$ T cells was determined by [^{3}H]thymidine uptake assay. (b) The impact of mDC_{EXO} stimulation of OT I $CD8^+$ T-cell proliferation by adding each of the neutralizing reagents, a mixture of neutralizing reagents together (mixed reagents), and a mixture of control antibodies and fusion proteins (control reagents) was assessed. $^*P < 0{\cdot}05$ versus cohorts without adding any neutralizing reagent (Student's *t*-test). One representative experiment of three is displayed.

T cells. Among them, EXO-pulsed mDC_{EXO} is the most efficient stimulator. To investigate the molecular mechanism involved in $CD8^+$ T-cell proliferation, a panel of reagents was added to the cell cultures. As shown in Fig. 3(b), the anti-MHC class I, anti-LFA-1 antibody, and CTLA-4/Ig could significantly inhibit the OT I $CD8^+$ T-cell proliferative response in the cocultures by 62%, 49% and 56% ($P < 0{\cdot}05$), respectively. A more effective inhibition of the proliferation of $CD8^+$ T cells, by 95%, was observed in the mixed reagents group ($P < 0{\cdot}05$), indicating that the $CD8^+$ T-cell proliferation is critically dependent on pMHC I/TCR specificity, and greatly affected by costimulation (CD80/CD28 and CD54/LFA-1).

EXO-pulsed DC activate $CD8^+$ T-cell proliferation *in vivo*

To assess whether DC pulsed with EXO_{OVA} can also stimulate $CD8^+$ T-cell proliferation *in vivo*, we performed kinetic studies using ELISPOT and tetramer staining assays.[33] As shown in Fig. 4(a,b), the OVA-specific and IFN-γ-secreting $CD8^+$ T-cell proliferative responses peaked at day 7 and then declined at day 9 after immunization with DC_{OVA}, EXO_{OVA}, mDC_{EXO} and $imDC_{EXO}$, respectively. EXO_{OVA} itself could only induce an average of 319 IFN-γ-secreting cells/10^6 splenocytes or 1·42% tetramer-positive $CD8^+$ T cells of the total $CD8^+$ T-cell population at day 7 after immunization, indicating that EXO_{OVA} can induce activation of naive antigen-specific $CD8^+$ T-cell responses *in vivo*, but to a much lesser extent compared with DC_{OVA} (504 IFN-γ-secreting cells/10^6 splenocytes and 2·88% tetramer-positive $CD8^+$ T cells). Interestingly, mDC_{EXO} induced the strongest $CD8^+$ T-cell responses (680 IFN-γ-secreting cells/10^6 splenocytes and 3·36% tetramer-positive $CD8^+$ T cells), indicating that EXO-pulsed mDC_{EXO} can efficiently prime naive $CD8^+$ T-cell responses *in vivo*. Our data also showed that both DC_{OVA}, mDC_{EXO} and $imDC_{EXO}$, but not EXO_{OVA}, could only stimulate OVA-specific $CD8^+$ T-cell proliferation (0·42%, 0·68% and 0·32% tetramer-positive $CD8^+$ T cells of the total $CD8^+$ T-cell population) in CD4 KO mice, indicating that DC_{OVA}, mDC_{EXO} and $imDC_{EXO}$ induce mainly $CD4^+$ T helper-dependent $CD8^+$ CTL responses, but that they also induce some $CD4^+$ Th-independent $CD8^+$ CTL responses.

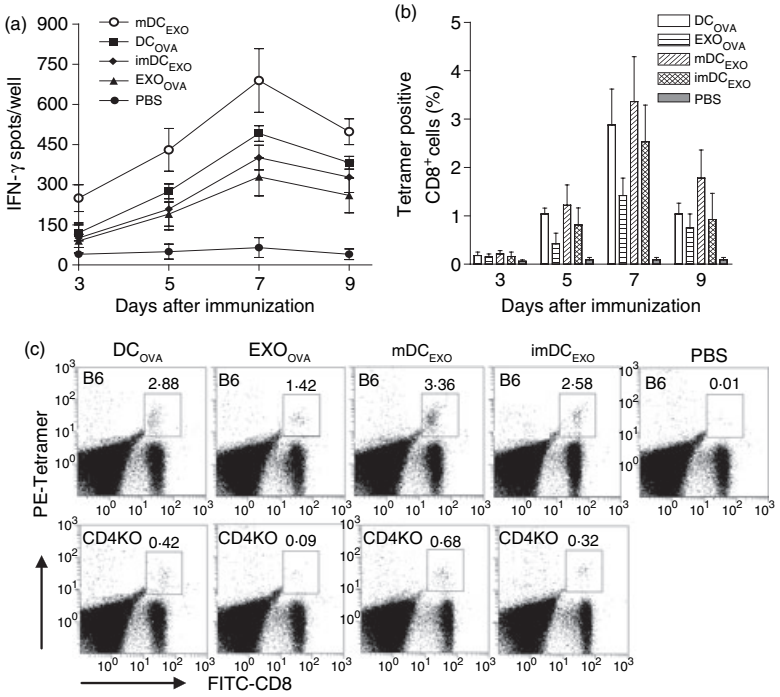

Figure 4. Stimulation of T-cell proliferation *in vivo*. (a) Mice were immunized i.v. with EXO_{OVA}, irradiated DC_{OVA}, mDC_{EXO} or $imDC_{EXO}$. After 3, 5, 7 and 9 days of immunization, the splenocytes were prepared from these immunized mice and assayed for IFN-γ-secreting $CD8^+$ T cells in response to OVA I stimulation in an Elispot assay. (b) After 3, 5, 7 and 9 days of immunization, tail blood samples were taken from these immunized mice and stained with phycoerythrin-conjugated (PE-) H-$2K^b$/OVA tetramer and FITC-conjugated anti-CD8 antibody. The expression of PE-H-$2K^b$/OVA tetramer-specific TCR and CD8 molecules was examined by flow cytometry. (c) A typical flow cytometric analysis of the tail blood samples taken from the wild-type C57BL/6 (B6) and CD4 KO mice 7 days after the immunization is shown. The value in each panel represents the percentage of tetramer-positive $CD8^+$ T cells versus the total $CD8^+$ T-cell population. The results presented are representative of four mice per group. One representative experiment of three is shown.

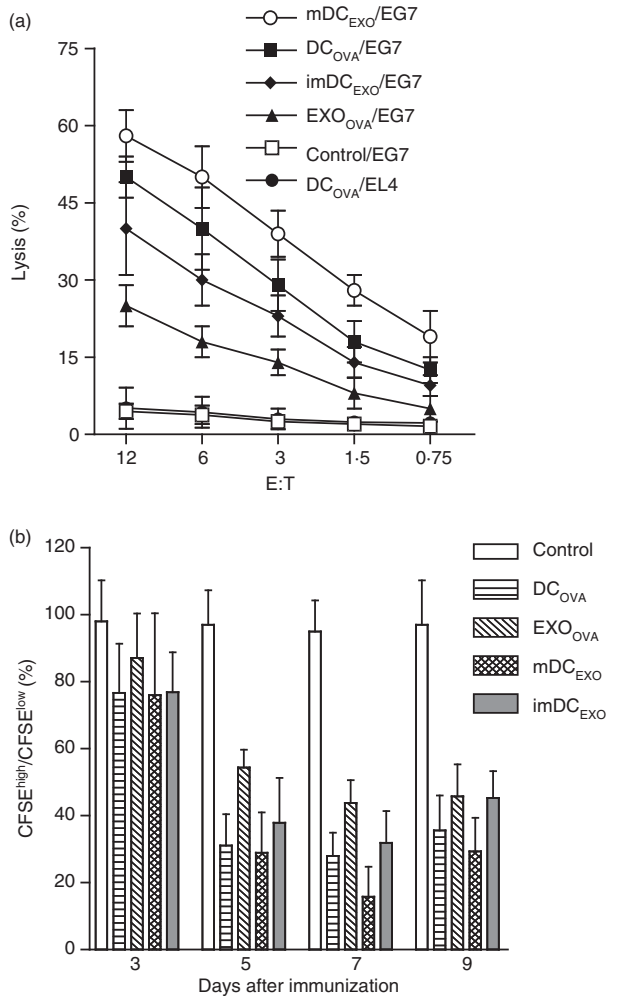

Figure 5. Development of antigen-specific CTL activities *in vitro* and *in vivo*. (a) *In vitro* cytotoxicity assay, naive OTI CD8$^+$ T cells (2×10^5 cells/ml) were stimulated for 3 days with EXO$_{OVA}$ (10 μg/ml) or irradiated (4000 rads) DC$_{OVA}$, mDC$_{EXO}$ and imDC$_{EXO}$ ($0{\cdot}6 \times 10^5$ cells/ml). These activated CD8$^+$ T cells were used as effector (E) cells, whereas ^{51}Cr-labelled EG7 or control EL-4 tumour cells were used as target (T) cells. Specific killing was calculated as: 100 × [(experimental c.p.m. – spontaneous c.p.m.)/(maximal c.p.m. – spontaneous c.p.m.)], where c.p.m. is counts per minute, as previously described. The data are presented as the % specific target cell lysis in a ^{51}Cr-release assay. Each point represents the mean of triplicate cultures. (b) In *in vivo* cytotoxicity assay, C57BL/6 splenocytes were harvested from naive mouse spleens and incubated with either high (3·0 μM, CFSE^{high}) or low (0·6 μM, CFSE^{low}) concentrations of CFSE, to generate differentially labelled target cells. The CFSE^{high} cells were pulsed with OVA I peptide, whereas the CFSE^{low} cells were pulsed with Mut 1 peptide and served as internal controls. These peptide-pulsed target cells were i.v. injected at a 1 : 1 ratio into the above immunized mice 3, 5, 7 and 9 days after immunization of EXO$_{OVA}$, DC$_{OVA}$, mDC$_{EXO}$ and imDC$_{EXO}$, respectively. After 16 hr, the spleens of immunized mice were removed and the residual CFSE^{high} and CFSE^{low} target cells remaining in the recipients' spleens were analysed by flow cytometry. One representative experiment of three is shown.

EXO-pulsed DC stimulate CD8$^+$ T-cell differentiation into CTL effectors *in vitro* and *in vivo*

In *in vitro* cytotoxicity assay, CD8$^+$ T cells activated by EXO$_{OVA}$ *in vitro* displayed killing activities against EG7 cells (25% killing; effector : target ratio, 12 : 1), but these were much weaker than those activated by DC$_{OVA}$, mDC$_{EXO}$ and imDC$_{EXO}$ (50%, 58% and 39%; effector : target ratio, 12 : 1) (Fig. 5a), respectively. No killing activities against its parental EL4 tumour cells were detectable, indicating that the killing activity of these CTLs is OVA specific. In the *in vivo* cytotoxicity assay, we adoptively transferred OVAI peptide-pulsed splenocytes that had been strongly labelled with CFSE (CFSE^{high}) as well as the control Mut1 peptide-pulsed splenocytes that had been weakly labelled with CFSE (CFSE^{low}) into the recipient mice that had been vaccinated with EXO$_{OVA}$, DC$_{OVA}$, mDC$_{EXO}$ and imDC$_{EXO}$, respectively. The peak of loss of CFSE^{high} target cells occurred on day 7 after immunization in all the groups tested (Fig. 5b). No CFSE^{high} target cell loss (> 2%) was observed in mice immunized with PBS. As expected, there was substantial loss of the CFSE^{high} cells in the immunized mice. Among them, the mice immunized with mDC$_{EXO}$ and EXO$_{OVA}$ had the highest (84%) and the lowest (57%) losses of the CFSE^{high} target cells, respectively (Fig. 5b), indicating that EXO-pulsed mDC$_{EXO}$ can most efficiently stimulate CD8$^+$ T cells differentiating into CTL effectors.

EXO-pulsed DC induce stronger immunity against lung tumour metastases

To investigate the induction of antitumour immunity, mice were i.v. immunized with EXO$_{OVA}$, DC$_{OVA}$, imDC$_{EXO}$ and mDC$_{EXO}$. Six days after the immunization, the immunized mice were i.v. challenged with BL6-10$_{OVA}$ tumour cells. As shown in experiment I of Table 1, all the mice injected with PBS had large numbers (> 100) of lung metastatic tumour colonies. EXO$_{OVA}$ vaccine only protected five of eight (63%) mice, as did the imDC$_{EXO}$ vaccine, whereas both DC$_{OVA}$ and mDC$_{EXO}$ vaccines induced complete immune protection against BL6-10$_{OVA}$ tumour challenge in all eight (100%) of the immunized mice. The specificity of the protection was confirmed with the observation that mDC$_{EXO}$ did not protect against BL6-10 tumours that did not express OVA, with all mice having large numbers (> 100) of lung metastatic tumour colonies after tumour cell challenge. The protective immunity derived from DC$_{OVA}$ and mDC$_{EXO}$ vaccines was still maintained in some CD4 KO mice, but was completely lost in CD8 KO mice, indicating that DC$_{OVA}$- and mDC$_{EXO}$-derived antitumour immunity is mediated by CD8$^+$ T cells. To compare the efficiency of antitumour immunity, different doses of DC$_{OVA}$ and mDC$_{EXO}$ were administered. As shown in experiment II of Table 1, mDC$_{EXO}$ vaccination at lower doses

Table 1. Exosome-targeted DC vaccine protects against lung tumour metastases

Vaccines	Tumour cell challenge	Tumour growth incidence (%)	Median no. lung tumour colonies
Experiment I			
DC_{OVA}	BL6-10_{OVA}	0/8 (0)	0
EXO_{OVA}	BL6-10_{OVA}	3/8 (37)	27 ± 6
mDC_{EXO}	BL6-10_{OVA}	0/8 (0)	0
$imDC_{EXO}$	BL6-10_{OVA}	2/8 (25)	16 ± 5
PBS	BL6-10_{OVA}	8/8 (100)	> 100
DC_{OVA}	BL6-10	8/8 (100)	> 100
mDC_{EXO}	BL6-10	8/8 (100)	> 100
DC_{OVA} (CD4KO)	BL6-10_{OVA}	2/8 (25)	15 ± 7
mDC_{EXO} (CD4KO)	BL6-10_{OVA}	1/8 (12)	13
DC_{OVA} (CD8KO)	BL6-10_{OVA}	8/8 (100)	> 100
mDC_{EXO} (CD8KO)	BL6-10_{OVA}	8/8 (100)	> 100
Experiment II			
$0{\cdot}5 \times 10^6$ DC_{OVA}	BL6-10_{OVA}	0/8 (0)	0
$0{\cdot}2 \times 10^6$ DC_{OVA}	BL6-10_{OVA}	2/8 (25)	15 ± 6
$0{\cdot}1 \times 10^6$ DC_{OVA}	BL6-10_{OVA}	4/8 (50)	28 ± 9
$0{\cdot}05 \times 10^6$ DC_{OVA}	BL6-10_{OVA}	8/8 (100)	55 ± 14
$0{\cdot}5 \times 10^6$ mDC_{EXO}	BL6-10_{OVA}	0/8 (0)	0
$0{\cdot}2 \times 10^6$ mDC_{EXO}	BL6-10_{OVA}	0/8 (0)	0
$0{\cdot}1 \times 10^6$ mDC_{EXO}	BL6-10_{OVA}	1/8 (12)	16*
$0{\cdot}05 \times 10^6$ mDC_{EXO}	BL6-10_{OVA}	3/8 (37)	17 ± 8*
PBS	BL6-10_{OVA}	8/8 (100)	> 100
Experiment III			
DC_{OVA}	BL6-10_{OVA}	8/15 (53)	35 ± 10
mDC_{EXO}	BL6-10_{OVA}	2/15 (13)	9 ± 7*
PBS	BL6-10_{OVA}	15/15 (100)	> 100
Experiment IV			
DC_{OVA}	BL6-10_{OVA}	0/8 (0)	0
mDC_{EXO}	BL6-10_{OVA}	0/8 (0)	0
$imDC_{EXO}$	BL6-10_{OVA}	0/8 (0)	0
PBS	BL6-10_{OVA}	8/8 (100)	> 100

In experiment I, wild-type C57BL/6, CD4 and CD8 KO mice ($n = 8$) were i.v. immunized with DC_{OVA}, EXO_{OVA}, mDC_{EXO}, $imDC_{EXO}$ or PBS. Six days after immunization, each mouse was challenged i.v. with OVA transgene-expressing BL6-10_{OVA} or wild-type BL6-10 tumour cells. In experiment II. wild-type C57BL/6 mice ($n = 8$) were i.v. immunized with different doses of DC_{OVA} and mDC_{EXO} ($0{\cdot}5 \times 10^6$–$0{\cdot}05 \times 10^6$ cells/mouse). Six days after immunization, each mouse was challenged i.v. with BL6-10_{OVA} tumour cells. The data pooled from three independent experiments were statistically analysed. *$P < 0{\cdot}05$ (non-parametric Mann–Whitney *U*-test)[48] versus cohorts of mice immunized with the same number of DC_{OVA}. In experiment III, wild-type C57BL/6 mice ($n = 15$) were first injected i.v. with BL6-10_{OVA} tumour cells. Five days after tumour injection, mice were then immunized i.v. with DC_{OVA} and EXO_{OVA}, respectively. *$P < 0{\cdot}05$ (non-parametric Mann–Whitney *U*-test) versus cohorts of DC_{OVA}. In experiment IV, wild-type C57BL/6 mice ($n = 8$) were i.v. immunized with DC_{OVA}, EXO_{OVA}, mDC_{EXO}, $imDC_{EXO}$ or PBS. Three months after immunization, each mouse was challenged i.v. with BL6-10_{OVA} tumour cells. The mice were killed 4 weeks after tumour cell challenge and the numbers of lung metastatic tumour colonies were counted. One representative experiment of three is shown.

($0{\cdot}05 \times 10^6$–$0{\cdot}2 \times 10^6$ cells/mouse) demonstrated more efficient protection than DC_{OVA}, although both of them at high dose ($0{\cdot}5 \times 10^6$ cells) showed 100% immune protection against BL6-10_{OVA} tumour, indicating that mDC_{EXO} can induce stronger antitumour immunity than DC_{OVA}.

EXO-pulsed DC eradicate established tumours

To investigate the therapeutic effect of EXO-pulsed DC on established lung tumour metastasis, mice were first injected with BL6-10_{OVA} tumour cells. After 5 days, the mice

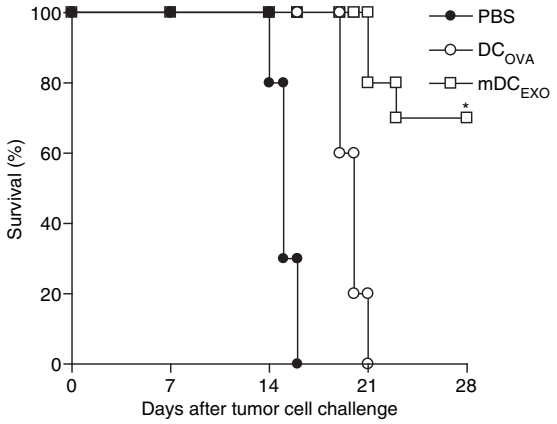

Figure 6. Therapeutic effect on established tumours. C57BL/6 mice ($n = 10$) were also s.c injected with $0{\cdot}3 \times 10^6$ BL6-10$_{OVA}$ tumour cells. After 4 days, when tumours became palpable (~3 mm in diameter), mice were s.c. immunized with irradiated DC$_{OVA}$ and mDC$_{EXO}$. Animal mortality was monitored daily. $^*P < 0{\cdot}05$ versus cohorts in DC$_{OVA}$ group (Log rank test).[46] The data are representative of two experiments with similar results.

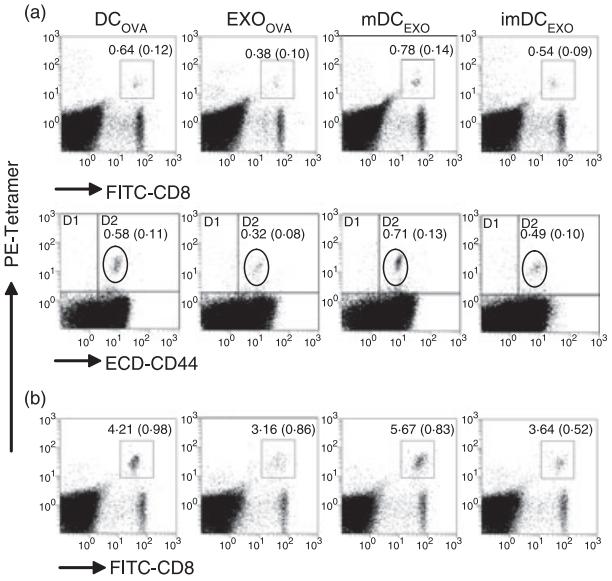

Figure 7. Development of antigen-specific CD8$^+$ memory T cells. (a) C57BL/6 mice were immunized with EXO$_{OVA}$, DC$_{OVA}$, mDC$_{EXO}$ and imDC$_{EXO}$, respectively. Three months later, tail blood samples were taken from these immunized mice and stained with phycoerythrin-conjugated (PE-) -H-2K^b/OVA tetramer and FITC-anti-CD8 antibody or ECD-anti-CD44 antibody, and analysed by flow cytometry. The PE-tetramer-positive T cells are also ECD-CD44-positive in each respective group assessed by flow cytometric sorting analysis. (b) The above immunized mice were boosted with DC$_{OVA}$. Four days after the boost, the recall responses were examined using staining with PE-H-2K^b/OVA tetramer and FITC-anti-CD8 antibody and analysed by flow cytometry. The value in each panel represents the percentage of tetramer-positive CD8$^+$ T cells versus the total CD8$^+$ T-cell population. The value in parenthesis represents the standard deviation. The results presented are representative of four separate mice per group. One representative experiment of three is shown.

were then immunized with DC$_{OVA}$ and mDC$_{EXO}$. As shown in experiment III of Table 1, 13 out of 15 (87%) mice with mDC$_{EXO}$ immunization were tumour-free, compared with only seven out of 15 (47%) mice cured in the DC$_{OVA}$ group, indicating that EXO-pulsed mDC$_{EXO}$ can more efficiently eradicate established lung tumour metastases than DC$_{OVA}$. To further confirm its enhanced therapeutic effect, we immunized the mice bearing s.c. palpable tumours (3 mm in diameter) with EXO-targeted DC and DC$_{OVA}$. As shown in Fig. 6, all the mice in the PBS control group died 16 days after tumour cell challenge. DC$_{OVA}$ vaccination retarded tumour growth, but did not protect mice from established tumour growth. All (10 out of 10) mice died of their tumours after 3 weeks of tumour cell inoculation. However, EXO-pulsed mDC$_{EXO}$ protected seven out of 10 mice from established tumour growth, indicating that not only can EXO-targeted mDC$_{EXO}$ more efficiently induce protective antitumour immunity than DC$_{OVA}$ vaccination, but they can also induce therapeutic effect on palpable tumours.

EXO-pulsed DC induce strong long-term OVA-specific CD8$^+$ T-cell memory

Active CD8$^+$ T cells can become long-lived memory T (Tm) cells after adoptive transfer *in vivo*.[34] Since mDC$_{EXO}$ stimulated CD8$^+$ T-cell differentiation into CTL effectors *in vitro* and *in vivo*, we then assessed whether these activated CD8$^+$ T cells can become long-lived Tm cells. As shown in Fig. 7(a), 3 months after the immunization, we detected 0·64%, 0·38%, 0·78% and 0·54% CD8$^+$ T cells expressing H-2K^b/OVA$_{257–264}$ tetramer-specific TCR in the peripheral blood of mice immunized with DC$_{OVA}$, EXO$_{OVA}$, mDC$_{EXO}$ and imDC$_{EXO}$, respectively. These OVA-specific CD8$^+$ T cells also express CD44, a Tm marker,[35] indicating that all these vaccines can induce the development of OVA-specific CD8$^+$ Tm cells. Among them, mDC$_{EXO}$ represent the strongest. To investigate the functionality of these CD8$^+$ Tm cells, the immunized mice were boosted with DC$_{OVA}$. The recall responses were examined using H-2K^b/OVA$_{257–264}$ tetramer staining on day 4 after the boost. As shown in Fig. 7(b), few OVA-specific CD8$^+$ T cells were detected in the peripheral blood of the mice, which were injected with PBS 3 months previously and boosted with DC$_{OVA}$ 4 days earlier, indicating that the primary proliferation of OVA-specific CD8$^+$ T cells is almost undetectable by DC$_{OVA}$ boost at that time-point. As expected, the number of CD8$^+$ T cells expressing H-2K^b/OVA$_{257–264}$ tetramer-specific TCR was expanded six- to seven-fold in the immunized mice after the boost, indicating that these CD8$^+$ Tm cells are functional. In another set of experiments, the above immunized mice were challenged with BL6-10$_{OVA}$ tumour cells 3 months after the immuniza-

tion. As expected, the control mice died of lung metastases. In contrast, mice immunized with mDC_{EXO}, $imDC_{EXO}$ and DC_{OVA} were tumour free (experiment IV of Table 1), confirming that these $CD8^+$ Tm cells remained functional.

Discussion

In recent years, EXO research has been stimulated by the finding that antigen presenting cells such as B lymphocytes and DC secrete EXO during exocytic fusion of multivesicular MHC class II compartments with the cell surface.[3,4] Formation of EXO occurs in MHC class II enriched compartments (MIIC) by macroautophagy of the internal membrane, then EXO are exocytosed by direct fusion of MIIC with plasma membrane. EXO from mature bone marrow-derived DCs display immunologically important molecules such as MHC class I and II, CD54 and costimulatory molecule CD86,[36] necessary for induction of immune responses. It has also been shown that CD54 on EXO from mature DC is critical for efficient naive T-cell priming.[37] However, EXO from immature spleen DC lacking immunostimulatory molecules such as CD40, CD86 and MHC II induced a protective response against the growth of a transplanted tumour, though they did not function in direct T-cell activation *in vitro*.[38] EXO-based vaccines, though they have been shown to induce antitumour immunity,[12–16] were still less effective by showing only the prophylatic immunity in animal models[12–16] or very limited immune responses in clinical trials.[17] In addition, the mechanism of EXO-mediated immunity *in vivo* is still poorly understood. The potential pathway of EXO-mediated immunity may be through uptake of EXO by the host imDC.

In this study, DC_{OVA}-derived EXO were systemically characterized by flow cytometry. We demonstrated that, in addition to the previously reported MHC class I and II, CD11b, CD54 and CD86 molecules,[5–7] EXO also expressed CD11c, costimulatory molecule CD80, chemokine receptor CCR7, mannose-rich C-type lectin receptor DEC205, TLR4 and TLR9. In addition, for the first time, we also demonstrated that EXO also expressed MHC class I/OVA I peptide (pMHC I) complexes and contained intracellular molecules such as MyD88 related to signal transduction, indicating that EXO carry all the immunologically important molecules of DC for induction of immune responses.

Membrane transfer has been abundantly reported in systems requiring or not requiring cell-to-cell contact.[39] Knight *et al*. have shown that DC acquire antigens from cell-free DC supernatants.[40] In this study, we demonstrated that EXO can be taken up by both mDC and imDC. The expression of immunologically important molecules such as MHC class II, CD40, CD54 and CD80 was all enhanced on DC after EXO uptake. The non-specific LFA-1/CD54 interaction between EXO and DC was involved in the EXO uptake, which is consistent with a previous report by Sprent *et al.*[32] In the immune system, C-type lectins and CLR have been shown to act as both the adhesion and the pathogen recognition receptors.[41] C-type lectins such as DEC205[42] have been demonstrated to mediate antigen uptake.[43] Interestingly, we found that the anti-DEC205 antibody can significantly reduce EXO uptake by DC, indicating that the interaction of C-type lectin and mannose-rich CLR may be involved in EXO uptake by DC. We then used a panel of monosaccharides in the blocking test. We found that both D-mannose and D-glucosamine significantly reduced EXO uptake by DC. Therefore, for the first time, we elucidated another important molecular mechanism of EXO uptake by DC [i.e. C-type lectin/mannose (glucosamine)-rich CLR interaction].

EXO_{OVA} derived from OVA protein-pulsed DC_{OVA} can stimulate OT I $CD8^+$ T-cell proliferation *in vitro*, which is also somewhat consistent with a previous report by Sprent *et al.*[32] In comparison, mature DC with EXO uptake (mDC_{EXO}) can more strongly stimulate $CD8^+$ T-cell proliferation and differentiation into effector CTL than immature DC with EXO uptake ($imDC_{EXO}$), tumour antigen-pulsed mature DC (DC_{OVA}) and EXO_{OVA}. It is possibly because of the higher level expression of MHC class II, CD40, CD54 and CD80 on mDC_{EXO} than on $imDC_{EXO}$ and OVA-pulsed DC_{OVA}. In the case of EXO vaccine, it needs DC adjuvant through EXO uptake by the host immature DC for induction of immune responses[14,20] and may thus be equivalent to our $imDC_{EXO}$ vaccine. In addition, EXO-pulsed mDC_{EXO} vaccine can further induce more effective OVA-specific CTL responses against OVA-expressing BL6-10_{OVA} tumour cells and antitumour immunity, as demonstrated in our established lung tumour metastasis and subcutaneous tumour models. Since tumour cell-derived EXO is a good source of tumour antigens,[10] EXO-pulsed DC vaccine may become a feasible vaccine for combating tumours by using EXO purified from a cancer patient's ascites,[12,44] which are then taken up by *in vitro*-activated DC derived from the patient's peripheral blood monocytes. Thus, EXO-pulsed DC vaccine may represent a novel and feasible EXO- and DC-based vaccine approach against tumours.

Taken together, our data showed that OVA protein-pulsed DC_{OVA}-derived exosomes (EXO_{OVA}) can be taken up by DC via LFA-1/CD54 and C-type lectin/mannose(glucosamine)-rich CLR interactions. EXO-pulsed mDC_{OVA} expressing a higher level of pMHC I and costimulatory CD40, CD54 and CD80 molecules can more efficiently stimulate naive OVA-specific $CD8^+$ T-cell proliferation *in vitro* and *in vivo*, and induce OVA-specific CTL responses, antitumour immunity and $CD8^+$ T-cell memory *in vivo* than EXO_{OVA} and DC_{OVA}. Therefore, the EXO-pulsed mDC_{OVA} may represent a new, highly

effective DC-based vaccine for the induction of antitumour immunity.

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